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## REVIEW

e-Xtra\*

## The Nodule Microbiome: N<sub>2</sub>-Fixing Rhizobia Do Not Live Alone

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### ABSTRACT

For decades, rhizobia were thought to be the only nitrogen-fixing inhabitants of legume nodules, and biases in culture techniques prolonged this belief. However, other bacteria, which are not typical rhizobia, are often detected within nodules obtained from soil, thus revealing the existence of a phytomicrobiome where the interaction among the individuals is not only complex, but also likely to affect the behavior and fitness of the host plant. Many of these nonrhizobial bacteria are nitrogen fixers, and some also induce nitrogen-fixing nodules on legume roots. Even more striking is the incredibly diverse population of bacteria residing within nodules that elicit neither nodulation nor nitrogen fixation. Yet, this community exists within the nodule, albeit clearly out-numbered by nitrogen-fixing rhizobia. Few studies of the function of these nodule-associated bacteria in nodules have been performed, and to date, it is not known whether their presence in nodules is

biologically important or not. Do they confer any benefits to the *Rhizobium*-legume nitrogen-fixing symbiosis, or are they parasites/saprophytes, contaminants, or commensals? In this review, we highlight the lesser-known bacteria that dwell within nitrogen-fixing nodules and discuss their possible role in this enclosed community as well as any likely benefits to the host plant or to the rhizobial inhabitants of the nodule. Although many of these nodule inhabitants are not capable of nitrogen fixation, they have the potential to enhance legume survival especially under conditions of environmental stress. This knowledge will be useful in defining strategies to employ these bacteria as bioinoculants by themselves or combined with rhizobia. Such an approach will enhance rhizobial performance or persistence as well as decrease the usage of chemical fertilizers and pesticides.

The fixation of atmospheric nitrogen (N<sub>2</sub>) into ammonia by bacteria is essential for plant productivity, especially in N-poor soils. About 60% of the fixed N on Earth results from biological nitrogen fixation whereas chemical fertilizers account for ca. 25% (Zahran 1999). The Green Revolution of the last century resulted in crops that produced higher yields. However, the improved crops relied heavily on chemical fertilizers particularly nitrate fertilizers, which resulted in ground water pollution and negative effects on human health (McCasland et al. 1985). Since the late 20th century, scientific research has focused mainly on plant biotechnology to improve crop productivity, but in this century, scientists are renewing interest in nitrogen-fixing microbes as well as the beneficial bacteria that act as plant growth-promoting rhizobacteria/bacteria (PGPR/PGPB). We propose that the rhizobia and the “other” bacteria act together as a community within the root nodule to facilitate plant health and survival, particularly under conditions of environmental stress.

The phytomicrobiome or plant microbiome is defined as all the microorganisms that colonize everything connected to the plant body, i.e., the rhizosphere and the phyllosphere, and includes all the directly associated endophytes and epiphytes (Quiza et al. 2015). Thus, the phytomicrobiome is a subset of the phytobiome, which has been described as plants, their environment, and the organisms that interact with them, and which together influence plant health and productivity (Leach et al. 2017). Taking a phytomicrobiome-focused perspective concerning the nodule and looking beyond the interaction of a legume with a single nitrogen-fixing species may help us better understand how to grow, fertilize, and protect crops in a sustainable way.

The nitrogen-fixing bacteria that comprise the majority of the microbial population of legume nodules, both  $\alpha$ -rhizobia (members of the *Alphaproteobacteria*, e.g., *Rhizobium* and *Bradyrhizobium*) and  $\beta$ -rhizobia (*Betaproteobacteria*, e.g., *Cupriavidus* and *Burkholderia* (reviewed by Gyaneshwar et al. 2011), are the best known and the most studied inhabitants of legume nodules. Even though  $\alpha$ - and  $\beta$ -rhizobia are evolutionary divergent, their symbiotic (*nod* and *nif*) genes are highly similar suggesting lateral transfer (Bontemps et al. 2010; Chen et al. 2003; De Meyer et al. 2016; Moulin et al. 2001). However, legume root nodules contain many other microbial residents. Figure 1 illustrates that in addition to rhizobia (Fig. 1A), F1 a mélange of soil microbes associate with roots (Fig. 1B to F), and many of them (Fig. 1C to F) inhabit the interior of nodules. With regard to fungi, the community in the legume nodule was found to

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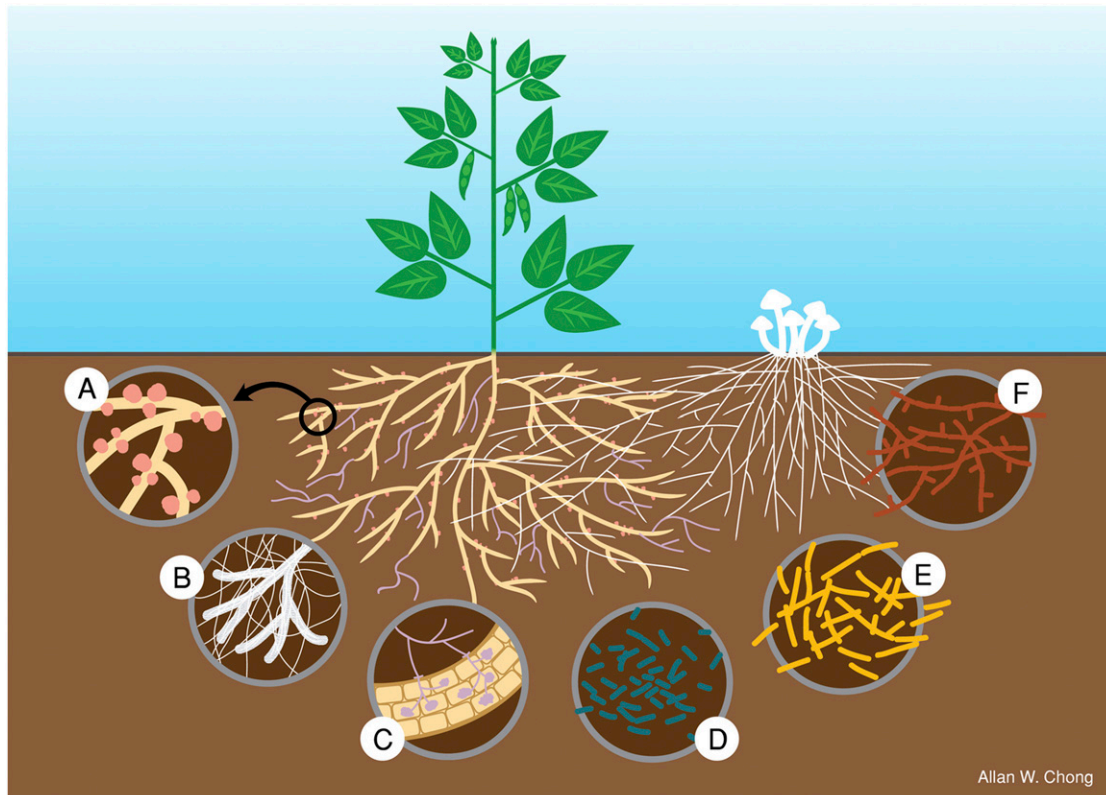
differ greatly from that found elsewhere in the plant and also from nonlegume plants, supporting the idea of a selected and curated microbiome in the nodule (Scheublin et al. 2004). Another study showed that inoculating plants with AM fungi changed the bacterial community and improved plant growth most likely because of improved shoot N, P, and K levels (Rodríguez-Caballero et al. 2017). Nevertheless, the most commonly isolated members of the legume nodule community outside of rhizobia consist of Gram-positive and Gram-negative bacteria, some of which have the capacity to fix  $N_2$  (Aserse et al. 2013; Deng et al. 2011; Muresu et al. 2008).

A vast literature on microbes other than rhizobia from a broad survey of legumes has accumulated starting with the earliest report made by Beijerinck (1888), who described the isolation of yellow-pigmented and brownish-colored *Bacillus*-like cells from nodules (cited in Fred et al. 1932). This account was soon followed by other nonrhizobia isolations from nodules resulting in additional publications from 1888 to 1928, describing *Bacillus megatherium* (*megaterium*) and bacteria resembling *Bacillus anthracis* or *Bacillus subtilis* as well as *Clostridium* and *Micrococcus* species. Beijerinck and van Delden (1902) were the first to publish the isolation from clover root nodules of *Bacillus radiobacter*, also known as *Agrobacterium radiobacter* and now as *Rhizobium radiobacter* (Young et al. 2001). *Rhizobium radiobacter* and *Rhizobium rhizogenes* are common inhabitants of legume nodules (Velázquez et al. 2013). Other early reports of bacteria isolated from nodules and roots of clover and soybean were those by

Phillipson and Blair (1957), Manninger and Antal (1970), and Sturz et al. (1997).

These other nodule-isolated microorganisms were originally called root nodule bacteria, which confused them with the nitrogen-fixing rhizobia (Sturz et al. 1997). They are now known as non-rhizobia endophytes (NRE) (De Meyer et al. 2015), nodule endophytes (Velázquez et al. 2013), or nodule-associated bacteria (NAB) (Rajendran et al. 2012). In this review, we will use the abbreviation NAB, which is broader in scope than the other two designations. Most NAB are generally nonpathogenic although some related mammalian pathogens, e.g., certain *Burkholderia* and *Staphylococcus* species and also *Bordetella avium*, have been isolated from nodules (Diouf et al. 2007; Provorov 2000; Rasolomampianina et al. 2005; Sturz et al. 1997; Xu et al. 2014). Also, nodules of *Hedysarum*, a forage legume, were found to harbor *Enterobacter cloacae*, *Enterobacter kobei*, *Escherichia vulneris*, *Pantoea agglomerans*, and *Leclercia adecarboxylata* (Muresu et al. 2008). Because it has long been known that coinoculation of rhizobia and other bacteria, particularly *Bacillus* species, promotes not only nodulation (Schwartz et al. 2013 and papers cited therein), but also N availability in sustainable agricultural systems (Rajendran et al. 2012), it is assumed that many of the nonpathogenic bacteria found within nodules or in plant tissues as endophytes could be safe and effective partners for enhancing nitrogen fixation in legumes (Sturz et al. 2000).

Nevertheless, many scientists are cautious about isolating NAB from legume nodules. For example, Vincent (1970) recommended



**Fig. 1.** Diagram of the belowground interactions of a nodulated legume with a variety of microbes. **A**, Enlarged view of nitrogen-fixing nodules on the plant's roots (circled). **B**, Ectomycorrhizal associations are often established with legume tree roots, but the fungi remain external. **C**, Arbuscular mycorrhizal fungi interact with legume roots utilizing the same symbiotic pathway as used by *Rhizobium*. **D**, Gram-negative bacteria in the soil, such as *Pseudomonas*, *Klebsiella*, and *Ochrobactrum* spp. are established in the rhizosphere and some species may even nodulate legumes. **E**, Gram-positive microbes, including *Bacillus*, *Paenibacillus*, *Lysinobacillus*, and others are found in the rhizosphere and also within nodules. **F**, Actinomycetes, for example, *Micromonospora*, *Streptomyces*, and the nitrogen-fixing *Frankia* enhance plant growth.

that old or desiccated nodules not be used for acquiring rhizobial suspensions because of the high frequency of “contaminants,” even if the nodules were handled carefully, surface-sterilized, and then rolled on nutrient agar to check for bacteria growth. Both Vincent (1970) and Somasegaran and Hoben (1994) emphasized the importance of authentication of rhizobial isolates from nodules and the rhizosphere, and for many years, bacterial cultures that differed in appearance or growth characteristics from bona fide *Rhizobium* species were thrown away. A good example of a bacterial genus that very likely was discarded in this manner is *Burkholderia*. Knowlton et al. (1980) described *Pseudomonas* (now *Burkholderia*) *cepacia* as a “helper” strain because it promoted nodulation of *Alnus* when it was coinoculated with the symbiotic *Frankia* strain CpII. A more recent handbook entitled *Working with Rhizobia* by Howieson and Dilworth (2016) describes isolation methods and techniques to ensure that obvious contaminants are not selected. The handbook also offers a more comprehensive treatment of the  $\beta$ -rhizobia.

Over the years, a vast number of bacteria other than rhizobia have been found in nodules. Surprisingly, some of these isolates also have *nif* and *nod* genes and elicit nitrogen-fixing nodules on legumes just like the  $\alpha$ - and  $\beta$ -rhizobia. In this review, we present an overview of the research about the nonrhizobial inhabitants of the legume root nodule, emphasizing the involvement of these organisms in nodule processes and symbiosis (where known), as well as their role in the phytomicrobiome, and ultimately their potential for crop production and protection. We first discuss those bacteria

that have acquired the ability not only to fix nitrogen, but also to nodulate legumes. By understanding the nodule microbiome, we may learn how to promote agricultural sustainability of legumes and other crops not only toward ensuring world food security, but also toward preserving the soil.

## RHIZOBIAL AND NONRHIZOBIAL BACTERIA THAT FIX NITROGEN AND NODULATE LEGUMES

The finding that nonrhizobial bacteria were isolated from within nitrogen-fixing nodules was not readily accepted at first. However, ever since the initial report of the isolation of a nodulating  $\beta$ -proteobacterial strain (Moulin et al. 2001), the number of publications dealing with such bacteria has increased exponentially and has become an area of active investigation (Table 1). New techniques used in strain characterization and an increase in the number of leguminous plant genera studied have expedited the description of new root-nodule microorganisms that are unrelated to the classical rhizobia. The genera that have been more thoroughly investigated are described below and in Table 1. Most studies, however, focus on microbes that can be cultured because genomic analyses have only recently been applied to nodule microbiomes. In addition, cultivatable strains are still required for use as agricultural inoculants.

**Phylum proteobacteria.  $\alpha$ -Proteobacteria.** *Agrobacterium*. Cummings et al. (2009) identified a nodulating strain isolated from nodules of *Sesbania cannabina* as *Rhizobium radiobacter*

**TABLE 1**  
Nonrhizobial nodule-inducing bacterial endophytes isolates from legume root nodules

Phylum/class	Bacterial genus	Legume host	<i>nod</i> gene similarity	References
<b>Alpha-Proteobacteria</b>				
	<i>Agrobacterium</i>	<i>Sesbania</i> , <i>Glycine</i>	<i>Ensifer/Rhizobium</i>	Cummings et al. (2009), Youseif et al. (2014)
	<i>Aminobacter</i>	<i>Anthyllis</i>	<i>Mesorhizobium symbiovar loti</i>	Maynaud et al. (2012)
	<i>Bosea</i>	<i>Ononis</i> , <i>Lupinus</i>	<i>Mesorhizobium</i>	De Meyer and Willems (2012), Rincón et al. (2008)
	<i>Devosia</i>	<i>Neptunia</i>	<i>Rhizobium tropici</i>	Rivas et al. (2002)
	<i>Methylobacterium</i>	<i>Crotalaria</i> , <i>Listia</i> , <i>Lotononis</i>	<i>Burkholderia tuberum</i>	Ardley et al. (2013), Madhaiyan et al. (2009), Renier et al. (2008), Sy et al. (2001)
	<i>Microvirga</i>	<i>Listia</i> , <i>Lupinus</i> , <i>Vigna</i>	<i>Rhizobium</i> , <i>Bradyrhizobium</i> , <i>Burkholderia</i>	Ardley et al. (2012, 2013), Zilli et al. (2015)
	<i>Ochrobactrum</i>	<i>Cytisus</i> , <i>Lupinus</i>	<i>Rhizobium</i>	Trujillo et al. (2006), Zurdo-Piñero et al. (2007)
	<i>Phyllobacterium</i>	<i>Ononis</i> , <i>Sophora</i>	<i>Mesorhizobium</i>	Baimiev et al. (2007), Jiao et al. (2015), Rincón et al. (2008), Valverde et al. (2005)
	<i>Shinella</i>	<i>Kummerowia</i>	<i>Rhizobium tropici</i>	Lin et al. (2008)
<b>Beta-Proteobacteria</b>				
	<i>Burkholderia</i>	Papilionoid and Mimosoid	<i>Burkholderia</i>	Dobritsa and Samadpour (2016), Estrada-de los Santos et al. (2013, 2016), Gyaneshwar et al. (2011), Moulin et al. (2001)
	<i>Cupriavidus</i>	<i>Mimosa</i>	<i>Burkholderia</i>	Andam et al. (2007), Bontemps et al. (2010), Chen et al. (2001, 2003, 2005), De Meyer et al. (2016)
<b>Gamma-Proteobacteria</b>				
	<i>Klebsiella</i>	<i>Arachis</i> , <i>Glycine</i> , <i>Vicia</i>	ND	Ibáñez et al. (2009), Ozawa et al. (2003), Youseif and Hirsch ( <i>personal communication</i> )
	<i>Pseudomonas</i>	<i>Hedysarum</i> , <i>Robinia</i>	<i>Mesorhizobium</i>	Benhizia et al. (2004), Shiraishi et al. (2010)
<b>Actinobacteria</b>				
	<i>Rhodococcus</i>	<i>Lotus</i> , <i>Anthyllis</i>	<i>Mesorhizobium</i>	Ampomah and Huss-Danell (2011)

(*Agrobacterium tumefaciens*) IRBG74, and Youseif et al. (2014) isolated agrobacterial strains carrying both *nod* and *nif* genes enabling them to elicit soybean nodulation upon reinoculation.

**Methylobacterium.** The genus *Methylobacterium* (*Methylobacteriaceae*) is best known for using methanol or other C1 compounds as a sole source of carbon for growth, but no association with plant root nodules had been described until a species of the genus was reported to nodulate *Crotalaria podocarpa*, *Crotalaria perrotteti*, and *Crotalaria glaucoides* (Sy et al. 2001). This report was followed by a thorough study in which the *nodDABCUIJHQ* cluster was amplified (Renier et al. 2008).

**Microvirga.** The first description of the genus *Microvirga* (*Methylobacteriaceae*) as an endosymbiont in legume root nodules was published in 2012. Three novel species (*Microvirga lotononidis*, *Microvirga lupini*, and *Microvirga zambiensis*) that nodulated *Listia angolensis* and *Lupinus texensis* were described (Ardley et al. 2012, 2013), and recently, another nodule-inducing strain from *Vigna unguiculata* (cowpea) was described and sequenced (Zilli et al. 2015).

**Phyllobacterium.** Poorly studied in terms of the family *Rhizobiaceae*, the genus *Phyllobacterium* contains a nodule-inducing species, *Phyllobacterium trifolii* (Valverde et al. 2005). This discovery was soon followed by the detection of several other novel species, all of which were very closely related phylogenetically and capable of nodulating different legumes (Baimiev et al. 2007). A *Phyllobacterium* gene with similarity to *nodC* was reported from isolates of *Ononis tridentata* root nodules (Rincón et al. 2008).

In summary, lateral gene transfer of *nod* and *nif* genes not only allowed non-*Rhizobiaceae* (albeit Rhizobiales) species to establish highly specific nitrogen-fixing symbioses with legumes, but these strains also are likely to have genes for plant growth-promoting (PGP) traits that are lacking in classical rhizobia (Andrews and Andrews 2017).

**$\beta$ -Proteobacteria.** *Burkholderia* (*Burkholderiaceae*) strains were recognized as PGPR/PGPB or biocontrol agents (BCA) (Raupach and Kloepper 1998). This line of research eventually led to the discovery of their nodule-inducing abilities (Moulin et al. 2001). *Burkholderia* species nodulate both Papilionoid and Mimosoid legumes and fix nitrogen as bacteroids within nodule cells (reviewed by Gyaneshwar et al. 2011). These bacteria also promote the growth of agronomically important plants, such as *Phaseolus vulgaris* (common bean) and cowpea (*Vigna unguiculata*), by producing or manipulating phytohormones, by providing nutrients, and via other mechanisms (Barka et al. 2000; Poupin et al. 2013; Sessitsch et al. 2005) as well as by acting as BCA (Barka et al. 2000; Thakkar and Patel 2015).

As more *Burkholderia* species were described and investigated, it became clear that at least two different lineages were evident. A split was proposed such that the pathogenic group would remain in the genus *Burkholderia* and a group that contained symbiotic and environmental species would be transferred into a new genus to be named *Caballeronia* (Estrada-de los Santos et al. 2013, 2016; Gyaneshwar et al. 2011). However, several of these species were instead placed into a new genus named *Paraburkholderia* on the basis of conserved sequence indels as well as the differences in G+C content (Sawana et al. 2014). Following the split between *Burkholderia* and *Paraburkholderia*, Dobritsa et al. (2016) reclassified *Burkholderia* species that were phylogenetically related to the environmental and symbiotic strains as *Paraburkholderia*, and then proposed *Caballeronia* as the genus name for this newly combined group (Dobritsa and Samadpour 2016).

*Cupriavidus* (*Burkholderiaceae*). *Ralstonia taiwanensis*, isolated originally from *Mimosa* nodules (Chen et al. 2001), and shown

to be a bona fide *Mimosa* symbiont (Chen et al. 2003), was renamed *Cupriavidus taiwanensis*. In addition to the beneficial effect of promoting legume growth by means of N<sub>2</sub> fixation, some *Cupriavidus* strains are resistant to heavy-metal contamination in soil, making them ideal candidates for use as bioinoculants in areas in need of remediation (Marques et al. 2013; Oves et al. 2010). Nodulating strains of *Cupriavidus* contain *nodA* (Chen et al. 2005) and other *nod* genes. The *Cupriavidus nod* genes are similar to each other and form a distinct clade suggesting that they most likely had a single origin from a neotropical (Mimosoid) *Burkholderia* ancestor (Andam et al. 2007; Bontemps et al. 2010; De Meyer et al. 2016).

Because of the relatedness to pathogenic bacteria of symbiotic strains of *Cupriavidus*, employing these bacteria as soil inoculants might affect human or plant health. This concern may affect the future use of *Cupriavidus* in agriculture, just as it has in the genus *Burkholderia* (U.S. Environmental Protection Agency 2003).

**$\gamma$ -Proteobacteria.**  $\gamma$ -Proteobacteria have only recently been found to have nodule-inducing representatives. The first report of  $\gamma$ -proteobacteria in association with legumes occurred when isolates from surface-sterilized root nodules of *Hedysarum* were analyzed with amplified ribosomal DNA restriction analysis and random amplified polymorphic DNA fingerprinting, but no rhizobia were detected (Benhizia et al. 2004). Only Enterobacteriales and Pseudomonadales bacteria were isolated, suggesting that these orders might be responsible for nodule induction. Later, *Pseudomonas* and *Burkholderia* spp. were reported to nodulate *Robinia pseudoacacia* (black locust), and *nodA* and *nodC* genes, which were similar to those of *Agrobacterium* and *Mesorhizobium*, were detected, suggesting lateral transfer (Shiraishi et al. 2010).

*Klebsiella* is a  $\gamma$ -proteobacterial species detected in in several legumes (Table 1). A *Klebsiella* strain isolated from *Vicia faba* nodules in Egypt and engineered to express GFP induced infection threads and formed bacteroids within symbiosomes of N<sub>2</sub>-fixing nodules of reinfected plants (Fig. 2). The strain's identity was verified by ribotyping and by sequencing several housekeeping genes as well as *nifH* and *nodC* (S. H. Youseif and A. M. Hirsch, personal communication).

**Phylum actinobacteria.** *Rhodococcus*. Strains from this genus were isolated from nodules of *Lotus corniculatus* and *Anthyllis vulneraria* in Sweden. All the strains isolated in this study harbored a *nodA* gene and induced nodules on *Lotus corniculatus* roots (Ampomah and Huss-Danell 2011).

## NON-NODULATING ROOT NODULE INHABITANTS

In addition to nodule-inducing and nitrogen-fixing bacteria, additional microorganisms inhabit the nodule even though they do not induce nodule formation. Just as in the case of the  $\beta$ -rhizobia, recognition of these nodule inhabitants as being potentially involved in the symbiosis has been slow in coming. Only the most studied NAB genera are described below and in Table 2, whereas the genera that are not found as frequently in root nodules are included in Supplementary Table S1.

**Phylum proteobacteria.** This phylum contains not only  $\alpha$ - and  $\beta$ -rhizobia, but also NAB that may fix N<sub>2</sub>, but do not induce nodulation on the roots of the host from which they were isolated (Velázquez et al. 2013).

**$\alpha$ -Proteobacteria.** One of the best-characterized PGPBs/PGPRs, *Azospirillum*, is commercialized as an inoculum in several countries, and proven to have growth enhancement properties for a number of crops, including legumes. The mechanisms it uses to promote plant growth include plant hormone synthesis, siderophore production, phosphate solubilization activity, biocontrol ability, nitrogen fixation, and many more (Glick et al. 1999).

*Azospirillum brasilense* fixes nitrogen in association with certain nonlegumes (Steenhoudt and Vanderleyden 2000). Coinoculation of *Azospirillum* sp. with nodule-forming bacteria had a synergistic effect that is in part related to the production of flavonoids that induced *nod* gene expression (Volpin et al. 1996). Studies have shown that coinoculation of PGP *Pseudomonas fluorescens* P-93 or *Azospirillum lipoferum* S-21 with *Rhizobium* onto *Phaseolus vulgaris* roots increased yield (Yadegari et al. 2008). An increase in nodule number and productivity has been described in several coinoculation studies in *Vicia*, common bean, alfalfa, and other legumes (Cassán and Diaz-Zorita 2016).

Another genus known as an excellent PGPR/PGPB and nitrogen fixer is *Gluconacetobacter*. Commercial inoculants based on *Gluconacetobacter diazotrophicus* (e.g., Ene-2; www.arbolab.com.ar/es/productos/2lv/prom.html) are available. To our knowledge, no studies have been made to assess the location of the bacteria (intranodular or not), but coinoculation with a commercial rhizobial strain resulted in increased soybean yields (Reis and Teixeira 2015).

Although some members of the bacterial genus *Ochrobactrum* induce nodulation, nonnodulating strains have been isolated from *Cicer arietinum*, *Glycyrrhiza uralensis*, *Cytisus scoparius*, and other legume nodules. *Pisum sativum* isolates have in vitro PGPR/PGPB properties such as phosphate solubilization activity and establish biofilms (Tariq et al. 2014). Heavy metal resistant isolates

have been used in consortia with nodule-forming bacteria and other PGPR/PGPB for phytoremediation of contaminated soils with *Lupinus luteus* as the accumulating plant. In this case, plant biomass increased whereas accumulation of heavy metals decreased, suggesting a possible protective effect of the bacterial inoculum (Dary et al. 2010).

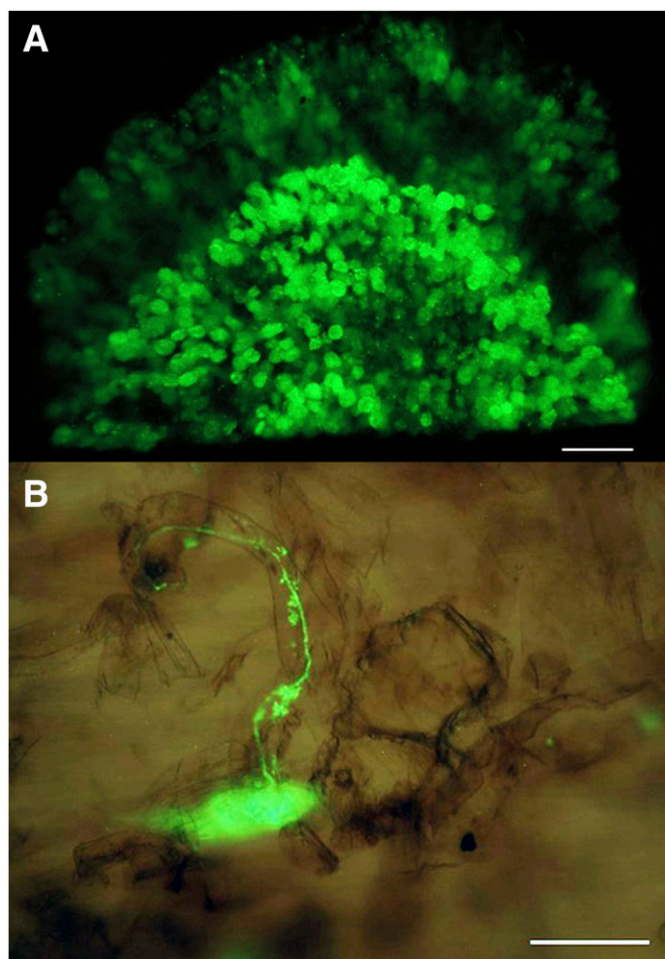
**β-Proteobacteria.** Among the β-proteobacteria, several representatives of the genus *Burkholderia* have been described as nodule inhabitants. Besides the nodule-inducing abilities of many *Burkholderia* members as mentioned in the previous section, isolates have been reported from root nodules of *Mimosa*, *Glycine*, *Arachis*, and *Lespedeza*. Although these isolates were unable to induce nodules upon reinoculation, they exhibited PGP ability suggesting a potential involvement in nodule function (Chen et al. 2014; Li et al. 2008, Palaniappan et al. 2010; Pandey et al. 2005).

Another interesting β-proteobacterial genus isolated from legume root nodules is *Variovorax*. Isolates have been obtained from root nodules of *Crotalaria incana* in Ethiopia. They showed a wide range of hydrolytic enzymatic activity, including the synthesis of lipase, cellulase, and protease (Aserse et al. 2013). In Australia, *Acacia salicina* and *Acacia stenophylla* nodules also contain strains closely related to *Variovorax paradoxus* (Hoque et al. 2011).

**γ-Proteobacteria.** *Klebsiella* and *Pseudomonas* have been reisolated from nodules containing both γ-proteobacteria and also *Ensifer adhaerens* (Pandya et al. 2013). In earlier studies, both genera were isolated from root nodules of *Arachis hypogaea* and then coinoculated with *Bradyrhizobium* sp. onto the same plant and reisolated, thereby confirming Koch's postulates (Ibáñez et al. 2009). The isolates from both genera, when coinoculated with rhizobia, increased nodule number and shoot dry weight. When compared with plants inoculated with rhizobia alone, this result suggested a growth-promoting role in the nodule ecosystem (Ibáñez et al. 2009).

Soybean-isolated strains of *Pantoea* from China were reported to have the ability to solubilize phosphate (Li et al. 2008). From studies of the microbiome of nodules in Ethiopia, *Pantoea* strains with cellulase activity were reported to inhabit *Phaseolus vulgaris* root nodules (Aserse et al. 2013). A study on coinoculating common bean with rhizobia and NAB, most likely γ-proteobacteria isolated from soils of western Kenya, reported enhanced plant growth over *Rhizobium*-only inoculated plants (Wekesa et al. 2016). Reducing the variation in yield of various crops across countries in Africa and other parts of the world via sustainable agriculture is one of the global challenges facing researchers today (Godfray et al. 2010). Coinoculations between rhizobia and indigenous NAB may offer solutions.

**Phylum firmicutes.** Reports of *Bacillus* strains isolated from root nodules are numerous (Table 2). Some studies show that *Bacillus* is the most abundant nonrhizobial genus found in nodules (Xu et al. 2014). Most of these isolates have been studied for their PGP properties, e.g., *Bacillus megaterium* in soybean increased plant nitrogen and nodule leghemoglobin when coinoculated with a nodulating strain (Subramanian et al. 2015). *Sophora alopecuroides* nodule isolates were reported to solubilize phosphate, produce siderophores and IAA, as well as have *nifH* although no evidence was given for nitrogenase activity (Zhao et al. 2011). In *Cicer arietinum* (chickpea), field studies demonstrated an increase in yield of 22% from coinoculating rhizobial and *Bacillus* strains compared with single inoculation treatments. Other studies have suggested the role of intranodular *Bacillus* in the induction of defense mechanisms, thereby increasing plant resistance to pathogens (Figueredo et al. 2014). Recently, sequencing of the genome of *Bacillus simplex* has been important for detecting genes that encode proteins likely to be involved in growth promotion of coinoculated peas such as genes for polyamine, lipopeptide, and hydrolytic enzyme synthesis, siderophore production, or phosphate solubilization (Maymon et al. 2015). *Bacillus simplex* strains were reported to have



**Fig. 2.** A *Klebsiella* strain isolated from the nodules of *Vicia faba* can renodulate the same plant species. **A**, Transverse section of a fava bean nodule showing GFP-labeled *Klebsiella* bacteria within nodule cells. Bar = 200 μm. **B**, GFP-labeled *Klebsiella* cells enter a curled root hair via an infection thread. Bar = 100 μm.

PGP activities and have been isolated as endophytes from various plants and also reisolated from pea nodules in coinoculation experiments with *Rhizobium leguminosarum* bv. *viciae* (Schwartz et al. 2013 and references therein). Deep analyses of NAB genomes may provide insights into understanding the involvement of many understudied microbes in plant growth and survival.

Representatives from the genus *Paenibacillus* are ubiquitous, and are isolated frequently from legume root nodules, although less frequently than *Bacillus* strains. For example, when bacteria were isolated from *Medicago sativa* nodules, *Paenibacillus* with chitinolytic activity was obtained (Lai et al. 2015). Other investigations have reported the potential use of *Paenibacillus* as a BCA, with in vitro antagonism against the fungal pathogens *Rhizoctonia*, *Fusarium*, and *Macrophomina*. PGP traits that were assayed include siderophore production, which is known to outcompete fungal iron chelating mechanisms (Senthilkumar et al. 2009).

**Phylum actinobacteria.** Species of the genus *Arthrobacter* were isolated from nodules of *Lespedeza* sp. growing in South Korea. Although the strain did not renodulate the host plant, it produced ACC deaminase as well as siderophores, both well-studied PGP traits (Palaniappan et al. 2010). An *Arthrobacter protophormiae* strain also enhanced plant growth under high salinity conditions, increasing nodule number and reducing salt stress (Barnawal et al. 2014). *Arthrobacter* strains have also been isolated from nodules of

berseem clover (*Trifolium alexandrinum*) growing in Egypt (S. H. Youseif, unpublished data).

The genus *Brevibacterium* was reported as a root inhabitant in native legumes from China (Xu et al. 2014). Subsequent studies on these same strains in *Cicer arietinum* showed that inoculation in the field significantly increased performance in all parameters measured, including nodule number and weight. At harvest, *Brevibacterium*-inoculated plants had significantly increased pod weight and number, as well as seed weight. Similar results were obtained when this strain was inoculated onto *Cajanus cajan*, suggesting that the strains are compatible with rhizobia, and synergistic with them (Gopalakrishnan et al. 2016).

Representatives of the genus *Micromonospora* have been isolated from different legume root nodules for several years (Table 2). Earlier, non-*Frankia* actinomycetes that were later identified as *Micromonospora* were isolated from nodules of *Casuarina* trees growing in Mexico (Niner et al. 1996). *Micromonospora* isolates have both antifungal (Martínez-Hidalgo et al. 2015) and PGP activity when inoculated, thus enhancing the growth and mineral nutrition of alfalfa (Martínez-Hidalgo et al. 2014b) and *Lupinus* (Cerdeña 2008). *Micromonospora* was also shown to be compatible with arbuscular mycorrhizal fungi (Martínez-Hidalgo et al. 2016).

Several studies have been made with respect to the genus *Streptomyces* having antifungal and plant growth enhancement abilities in association with roots or rhizosphere (Schrey and

**TABLE 2**  
Nonnodulating bacterial endophytes isolates from legume root nodules

Phylum/class	Bacterial genus	Legume host/coinoculation studies	References
<b>Alpha-Proteobacteria</b>			
	<i>Azospirillum</i>	<i>Trifolium</i> , <i>Phaseolus</i> , <i>Vicia</i> , <i>Medicago</i>	Cassán and Diaz-Zorita (2016), Plazinski and Rolfe (1985), Yadegari et al. (2008)
	<i>Gluconacetobacter</i>	<i>Glycine</i>	Reis and Teixeira (2015)
	<i>Ochrobactrum</i>	<i>Cicer</i> , <i>Glycyrrhiza</i> , <i>Pisum</i> , <i>Lupinus</i> , <i>Vigna</i>	Dary et al. (2010), Faisal and Hasnain (2006), Li et al. (2016), Tariq et al. (2014)
	<i>Methylobacterium</i>	<i>Arachis</i>	Madhaiyan et al. (2006)
<b>Beta-Proteobacteria</b>			
	<i>Burkholderia</i>	<i>Mimosa</i> , <i>Glycine</i> , <i>Arachis</i> and <i>Lespedeza</i>	Chen et al. (2014), Li et al. (2008), Palaniappan et al. (2010), Pandey et al. (2005), Raupach and Kloepper (1998)
	<i>Variovorax</i>	<i>Crotalaria</i> , <i>Acacia</i>	Aserse et al. (2013), Hoque et al. (2011)
<b>Gamma-Proteobacteria</b>			
	<i>Klebsiella</i>	<i>Vigna</i> , <i>Arachis</i>	Ibáñez et al. (2009), Pandya et al. (2013)
	<i>Pseudomonas</i>	<i>Vigna</i> , <i>Arachis</i>	Ibáñez et al. (2009), Pandya et al. (2013)
	<i>Pantoea</i>	<i>Mimosa</i> , <i>Lathyrus</i> , <i>Lotus</i> , <i>Medicago</i> , <i>Melilotus</i> , <i>Robinia</i> , <i>Trifolium</i> , <i>Vicia</i> , <i>Phaseolus</i>	Aserse et al. (2013), De Meyer et al. (2015), Lammel et al. (2013), Wekesa et al. (2016)
<b>Actinobacteria</b>			
	<i>Arthrobacter</i>	<i>Lespedeza</i> , <i>Pisum</i> , <i>Trifolium</i>	Barnawal et al. (2014), Palaniappan et al. (2010), Youseif and Hirsch (unpublished data)
	<i>Brevibacterium</i>	<i>Cicer</i> , <i>Cajanus</i>	Gopalakrishnan et al. (2016), Xu et al. (2014)
	<i>Micromonospora</i>	<i>Lupinus</i> , <i>Pisum</i> , <i>Medicago</i> , <i>Casuarina</i>	Carro et al. (2012), Martínez-Hidalgo et al. (2014a), Niner et al. (1996), Trujillo et al. (2006)
	<i>Streptomyces</i>	<i>Pisum</i> , <i>Cicer</i>	Sreevidya et al. (2016), Tokala et al. (2002)
<b>Firmicutes</b>			
	<i>Bacillus</i>	<i>Oxytropis</i> , <i>Cicer</i> , <i>Glycine</i> , <i>Calycotome</i> , <i>Sophora</i> , <i>Pisum</i>	Saini et al. (2015), Schwartz et al. (2013), Subramanian et al. (2015), Wei et al. (2015), Zakhia et al. (2006), Zhao et al. (2011)
	<i>Paenibacillus</i>	<i>Medicago</i> , <i>Cicer</i> , <i>Lupinus</i> , <i>Prosopis</i>	Carro et al. (2013), Carro et al. (2014), Lai et al. (2015), Valverde et al. (2010)

Tarkka 2008). More recent studies described the PGP properties of *Streptomyces* strains in greater detail (Sreevidya et al. 2016). This genus produces numerous secondary metabolites that could be used in plant nutrition and defense and should be considered as an asset for agricultural applications as a plant growth promoter as well as for biological control (Rey and Dumas 2017).

## DO NONRHIZOBIAL BACTERIA RESIDING WITHIN THE NODULE INFLUENCE THE SYMBIOSIS?

It is clear from the vast array of publications mentioned both in the text and in Tables 1 and 2 that many nonrhizobial bacteria are resident within legume nodules and that they possess a variety of PGP traits. Do these nonrhizobial residents have a function in the nodule and if so, what is it? Although a large number of investigators have coinoculated soil isolates and species of *Rhizobium*, far fewer studies have coinoculated NAB and rhizobia. An early report demonstrated that coinoculating *Rhizobium leguminosarum* bv. *trifolii* with the clover nodule isolates *Bacillus insoluitis*, *Bacillus brevis*, and *Agrobacterium rhizogenes* A promoted plant growth and nodulation compared with inoculating with *Rhizobium* alone (Sturz et al. 1997). In a more recent study, 17 different NAB were isolated from *Trigonella foenum-graecum* (fenugreek) nodules, and two isolates, both species of *Exiguobacterium*, enhanced plant growth when coinoculated with *Sinorhizobium meliloti* (Rajendran et al. 2012).

Numerous examples of coinoculations between *Bacillus* and rhizobial species have been published and in many cases, the double inoculation enhances legume growth. For example, coinoculation of *Bradyrhizobium* with *Bacillus subtilis* and *Bacillus thuringiensis* strains isolated from soybean nodules enhanced soybean growth under both greenhouse and field conditions (Bai et al. 2002). However, *Bacillus thuringiensis* strains were more effective. Similarly, *Bacillus thuringiensis* strain KR1, which was isolated from the wild legume *Pueria thunbergiana* (kudzu), promoted soybean root length, nodule number, and shoot dry weight, over the controls when coinoculated with *Bacillus japonicum* SB1 (Mishra et al. 2009). Auxin synthesis (Mishra et al. 2009) and the production of a class IId bacteriocin (Lee et al. 2009) by the *Bacillus thuringiensis* strains have been proposed as the stimulus for plant growth promotion.

In some cases, coinoculation experiments introduced a specific function into the microbial interaction that resulted in growth enhancement. Egamberdieva et al. (2010) coinoculated *Galega orientalis* Lam. with root-colonizing *Pseudomonas* species (*Pseudomonas trivalis* strain 3Re27 and *Pseudomonas extremorientalis* strain TSAU20) that synthesize IAA and *Rhizobium galega* bv. *orientalis*, which does not. The coinoculated plants exhibited enhanced root and shoot mass. However, *Pseudomonas trivalis* 3Re27, which was isolated originally from the potato endosphere and synthesizes cellulase, promoted nodule number in coinoculation experiments. It also increased N content, by 20% for roots and 52% for shoots, compared with *Rhizobium galega* alone and to the coinoculation between *Rhizobium galega* and the other *Pseudomonas* strain. Because the major difference between the two *Pseudomonas* strains is cellulase production, it was concluded that the supplementary production of this cell-wall degrading enzyme promoted nodulation. Indeed, *Rhizobium* cellulase is essential for entry of the nodulating bacteria into the host. Knockout mutants of the gene encoding the cellulase CelC2 in *Rhizobium leguminosarum* bv. *trifolii* ANU843 abolished infection thread formation (Robledo et al. 2008).

Ibáñez et al. (2009) performed coinoculation experiments on peanut with nodule-associated species of *Pseudomonas*, *Enterobacter*, and *Klebsiella* together with *Bradyrhizobium*. Although all three NAB species upon inoculation increased shoot dry weights over the uninoculated control when they were the sole inoculum, a significant gain in dry weight in the coinoculation trials occurred

when *Enterobacter* was the coinoculation partner. Although the *Klebsiella* isolates have *nif* genes (Ibáñez et al. 2009; Idris et al. 2015), they did not enhance plant growth. In contrast, some *Enterobacter* species are reported to synthesize auxin, and coinoculations between *Enterobacter* isolated from roots and chickpea rhizobia (*Mesorhizobium* sp.) enhanced nodulation and shoot/root biomass over the uninoculated and singly inoculated controls (Mirza et al. 2007).

We have isolated a large number of NAB from alfalfa nodules; the vast majority are firmicutes and actinomycetes (Martínez-Hidalgo et al. 2014b; P. Martínez-Hidalgo, E. Veliz-Madina, and A. M. Hirsch, unpublished data). Most of these bacteria are cellulase and chitinase producers, and preliminary tests indicate that coinoculation between some of the nodule isolates and *Sinorhizobium meliloti* enhance alfalfa biomass, nodule number, and N, P, and K levels. Some of the isolates also primed a defense response in tomato (Martínez-Hidalgo et al. 2015) supporting the use of these nodule inhabitants as bioinoculants. However, much more research on pairing rhizobia and NAB is needed.

## THE LEGUME PHYTOMICROBIOME

Investigations on phytomicrobiomes, particularly the phyllosphere and rhizosphere microbiota have uncovered considerable information, particularly in *Arabidopsis*, using metagenomic, transcriptomic, proteomic, and metabolomic analyses (reviewed by Müller et al. 2016). Also well-studied are the *Arabidopsis* phytomicrobiome responses to biotic and abiotic stresses as well as the changes that occur during a plant's development from seedling stage to maturity (Chaparro et al. 2014) and flowering (Wagner et al. 2014). Although soil type and plant root exudates influence the microbiota, host genetics plays an important part in the configuration of the plant microbiome as well (Carvalhais et al. 2015). Thus, the interaction between plants and their microbiome is not only intimate, but also mutually influenced (Hunter 2016).

Fewer studies have been made of legume microbiomes, especially of the nodule. A correlation was made in the genetic structure of the symbiotic bacterial and fungal communities with developmental stage of the model legume *Medicago truncatula* (Mougel et al. 2006). The mycorrhizal community was hardly altered in contrast to the bacterial community, which changed at each vegetative stage of the plant. During the first flowering stages, both communities changed, but at pod maturation, the bacterial community returned to the composition exhibited at the vegetative stages.

For *Trifolium pratense*, a combined root and nodule microbiome was prepared and investigated using cultivation and high throughput sequencing methods (Hartman et al. 2017). Rhizobia made up 70 or 78% of the root microbiome for the cultivation-independent or cultivation-dependent methods, respectively. Compared with *Arabidopsis*, a significant number (200) of bacteria could be cultured; this number corresponded to about 20% of the microbiome. Besides rhizobia, the cultured microbes included the proteobacteria *Pseudomonas*, *Stenotrophomonas*, and *Janthinobacterium*, and several actinobacteria, namely *Microbacterium*, *Micrococcus*, and *Micromonospora*. Single inoculation experiments without rhizobia and with the three PGPR/PGPB genera—*Pseudomonas*, *Janthinobacterium*, and *Microbacterium* resulted in an increase in shoot fresh weight. In contrast, inoculation with a *Flavobacterium* isolate reduced *T. pratense* growth. When coinoculation experiments were performed with rhizobia and all four isolates together (*Pseudomonas*, *Janthinobacterium*, *Microbacterium*, and *Flavobacterium*), shoot growth was restored, suggesting that the negative effect of *Flavobacterium* on plant growth was counteracted by one or more of the other bacteria (Hartman et al. 2017).



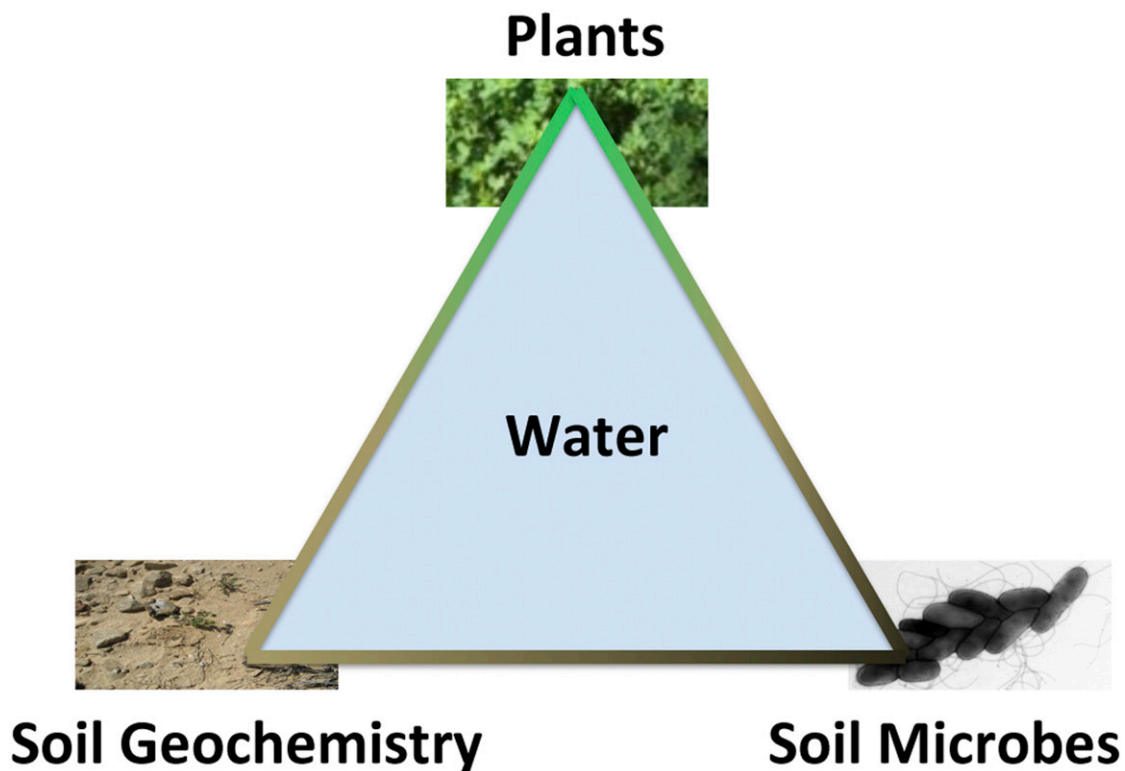
Several articles describe the rhizosphere, root, and nodule microbiomes of *Lotus* species, especially *L. japonicus*, an important model for the study of leguminous plants that develop determinate (spherical) nodules. For example, Unno and Shinano (2013) used metagenomics to understand rhizosphere microbiome structure and function in *L. japonicus* where phytic acid was being used as the sole phosphate fertilizer. The results showed that the bacterial community in this soil had a large number of phytase-producing bacteria and that some of these had PGP activity. In a more recent study, bacterial communities from the rhizosphere and from within nodules of wild-type *L. japonicus* and nodulation-impaired mutant plants were compared. Results strongly suggested that the plant's microbiome is influenced by the host's ability to accommodate beneficial bacteria and also illustrated how plant genotype influences the microbiome. The roots and rhizospheres of the early nodulation pathway mutants supported a different bacterial population than the wild-type plants, suggesting that the association between host and microbiome did not depend solely on N<sub>2</sub>-fixing rhizobia, but also required a direct connection between a symbiotically linked bacterial community that resides in the rhizosphere (Zgadzaj et al. 2016).

Natural nodule microbiomes, i.e., those collected directly from the field, remain under-investigated. An exception is *V. unguiculata* (cowpea), where early studies showed that nine different cowpea genotypes collected from fields in different parts of Africa had diverse bradyrhizobial strains residing in nodules based on intergenic-spacer restriction length polymorphism analysis, and the nodules also accumulated different amounts of fixed N. However, greater *Bradyrhizobium* diversity was observed in plants growing in a South African soil compared with soils from Ghana and Botswana (Pule-Meulenberg et al. 2010). A study of the nodule microbiomes of two different cowpea genotypes growing in Brazil reported that bacterial communities were influenced more by soil type than plant

genotype (Leite et al. 2017). The dominant nodular inhabitant consisted of diverse *Bradyrhizobium* OTUs, but also OTUs of  $\alpha$ - and  $\gamma$ -proteobacteria, bacteroidetes, actinobacteria, and firmicutes were detected although the latter was found only in ultisol samples. On the other hand, flavobacteria and actinobacteria were detected in both ultisol and vertisol samples.

A recent publication described five priorities for research into plant microbiomes for sustainable agriculture: (i) to develop model host-microbiome systems for crop plants and noncrop plants with associated microbial culture collections and reference genomes, (ii) to define core microbiomes and metagenomes in these model systems, (iii) to elucidate the rules of synthetic, functionally programmable microbiome assembly, (iv) to determine functional mechanisms of plant-microbiome interactions, and (v) to characterize and refine plant genotype-environment-microbiome-management interactions (Busby et al. 2017). We applaud the authors' efforts and particularly the comments regarding open lines of communication with farmers. However, we would like to recommend the addition of two items to this priority list. We believe it important to consider phyto-microbiomes from a global perspective, and thus include the studies of microbiomes in countries that in the past relied heavily on the 20th century Green Revolution. Strategies for increasing food production are often highly site-specific, and thus it will be important to focus on areas of the world with the most degraded soils because many of them will undergo the largest increases in population (Godfray et al. 2010). Nitrogen fixation and PGP microbes are more likely to be needed in places that utilize legume intercropping, agroforestry, legume cover crops, and/or do crop rotation with legumes.

Second, will a core microbiome developed in a model host-microbe system in the United States and Europe for a limited number of plants and soil types be utilizable for certain areas of Africa and Asia, where much of the soil is nutrient-poor, drought-stricken, contaminated with heavy metals, saline, and/or over-cultivated?



**Fig. 3.** The interactions of plants, unconsolidated mineral or organic material, and microbes, all of which require water to form soil, is depicted in this triangle. Not shown is the animal component.

To grow enough food on arid, eroded, or saline soils, agriculture relies heavily on nitrogen-fixing bacteria, biofertilizers, manure, and when affordable or transportable, chemical fertilizers, and often all of the above. Perhaps, a better strategy would be to analyze the phytomicrobiomes of a variety of plants, particularly legumes growing in many different soil types. The lessons learned from the host specificity studies on legume–rhizobia interactions should stimulate us to abandon the “one size fits all” approach. Thus, attention needs to be paid to the microbiomes of legume root nodules especially in their native environments. By doing so and learning how environmental as well as host factors affect the nodule microbial communities, we can begin to engineer consortia of microbes that better match the host and its surroundings. As an example, Ellis (2017) maintains that the study of microbiomes of diseased plants instead of healthy ones is more likely to uncover bacteria that function as BCA. Thus, core microbiomes need to be elucidated for major crops from diverse agricultural regions of the world because abiotic factors, soil geochemistry, and water availability all have a powerful influence on plant diversity and microbial populations. Efforts to learn more about indigenous rhizosphere microbiomes are being pursued in several countries, including Pakistan (Mukhtar et al. 2016), Cameroon (Sarr et al. 2016), and Sudan (Idris et al. 2015). It will also be interesting to determine whether various microbes are conserved in similar environments.

## CONCLUSIONS

Restoration ecology for the recovery of landfills, eroded soils, and damaged ecosystems is analogous to using fecal microbial therapy (FMT) on patients suffering from *Clostridium difficile* infection (CDI) brought about by overuse of antibiotics that kill the normal gut flora (McFarland 2016). Similar to the effect of long-term antibiotic use, decades of over-fertilization, pesticide accumulation, and over-tilling have depleted the soil of its beneficial organisms, which play an essential role in maintaining plant health (Berendsen et al. 2012) as well as soil fertility. Recent results showed that applying soil from natural ecosystems to overused agricultural soil restored fertility after 6 years (Wubs et al. 2016). Likewise, planting native legume trees in combination with growth-promoting bacteria, AM fungi, and added compost in eroded Sonora Desert soil resulted in soil improvement and tree growth after 30 months (Bashan et al. 2012). Both of these restoration efforts were successful, but in contrast to FMT, they also required considerable time as well as repositioning of macro-organisms and/or soil, both of which are laborious and expensive.

Moreover, although the gut microbiome serves as an important model for phytomicrobiome research, few overlaps in microbial composition exist between animal and plant microbiota (Hacquard et al. 2015). However, many of the functions, which the microbes perform in the service of the host, are conserved (Berendsen et al. 2012). Finding the right bacteria that restore plant growth in degraded soils will take as concerted an effort as the ongoing research to find the optimal combination of microbes for restoring the health of the human microbiome in the case of CDI.

Investigating the consortia of organisms within root nodules and selecting for beneficial microorganisms that can be used as inoculants for growth promotion is a logical extension of the studies described in this review, and efforts are already being made in this direction (Quiza et al. 2015). Mining root nodules will make it easier to find compatible microbial partners that significantly enhance plant, especially legume, growth in degraded soils. So far, mostly soil-isolated microbes have been used for PGPR/PGPB formulations. However, studies of the nodule microbiome will better suggest which microbes are superior for developing inocula.

Also, the issues of soil type and plant genotype are more readily addressed by studying nodule microbiomes from specific environments. Specificity between a legume and its rhizobial partner is well known, and it will be essential to learn whether similar patterns exist for PGPR/PGPB inocula. Until we know exactly what their functions are in the nodule and how they interact with rhizobia, we cannot know the final outcome. Currently, we empirically test numerous rhizobial–PGPR/PGPB combinations for the best plant growth. We can refine this approach by learning more about crucial biochemical pathways, microbe–microbe interactions, and plant–microbe interactions. This knowledge is rapidly expanding and will enable us to make predictions not only in terms of PGPR/PGPB relationships with specific legumes and their symbiotic partners, but also with regard to the different soil types in which they grow. The design of methods for coinoculation of the bacterial partners and the development of formulated inoculants will be essential for achieving agricultural sustainability. Considerable progress has already been made on both fronts (Bashan et al. 2016).

The relationship between plants, soil geochemistry, microbes, and of course, water, which permits these components to interact, can be visualized as a triangle (Fig. 3). This triangle is similar in concept to a disease triangle, but unlike the latter, it represents beneficial interactions between animate and inanimate inputs (Fig. 3). If the equilibrium between the constituents becomes unbalanced, Earth’s skin of fertile topsoil, along with the agriculture it sustains, erodes, causing civilizations to crumble (Montgomery 2007). Mesopotamia (modern-day Iraq and parts of Syria and Turkey), the once fertile “land between the rivers,” and the Nile Delta were the original cradles of western human civilization. Their collapses resulted in a serious destabilization of human activity that continues to this day. Understanding the phytomicrobiome, which is one-third of the triangle (Fig. 3), is essential not only for preserving soil health and promoting plant growth, but also for maintaining our planet and its inhabitants. Investigating the unique microbiomes of a diversity of nitrogen-fixing nodules to find potentially useful inoculants will enable us to achieve these goals.

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## **AUTHOR QUERIES**

### **AUTHOR PLEASE ANSWER ALL QUERIES**

Q:1 **AUTHOR:** Please define AM in AM fungi?

